EXHIBIT A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Audrey Goddard et al.	Examiner:	Lorraine Spector
Serial No.:	09/202,054	Group Art Unit:	1647
Filed:	December 7, 1998	Docket:	G&C 669.23-US-WO
Title:	HUMAN TOLL HOMOLOGUES		

DECLARATION OF J. FERNANDO BAZAN UNDER 37 C.F.R. § 1.132

I, J. FERNANDO BAZAN, declare as follows:

1. I am currently employed as a Senior Scientist at Genentech, Inc. Prior to my employment at Genentech, Inc., I was employed at DNAX Research Institute. I hold a Ph.D. in Biophysics and have been working in the field of technology relating to Toll proteins for over 10 years. A copy of my Curriculum vitae is included herein as Attachment A.

2. I understand that the above-referenced patent application was filed by Genentech, Inc. on December 7, 1998 ("the '054 application"). I was provided a copy of the '054 application by Genentech's patent attorney, and I have reviewed the '054 application.

3. The '054 application describes that a BLAST and FastA sequence alignment analysis showed that the polypeptide referred to as PRO285 is a human analogue of the Drosophila Toll protein, and is homologous to cytoplasmic domains of human interleukin 1 receptor and the following human Toll proteins: Toll1 (DNAX# HSU88540_1), Toll2 (DNAX# HSU88878_1), Toll3 (DNAX# HSU88879_1), and Toll4 (DNAX# HSU88880_1)". See, e.g. Figure 7B and page 40, lines 19-23, of the '054 application.

4. The '054 application also describes that specific regions of PRO285 homology include a 13 residue intracellular segment that is associated with NF-κB activation in Toll-like receptor 2 and Interleukin-1 receptor (as shown in Figure 7B and described at page 7, lines 8-23, of the '054 application). A sequence alignment showing this region of homology in the Interleukin-1 receptor, the Toll-like receptor 2 and PRO285 is provided herein as Attachment B. As shown in Attachment B, 9 of

the 13 residues in this region of PRO285 are either identical to or are conservative amino acid substitutions of the corresponding residues in the Interleukin-1 receptor and/or Toll-like receptor 2.

5. The '054 application describes that that 13 amino acid region is crucial for receptor mediated signaling and NF-κB activation in both the interleukin-1 receptor and the Toll-like receptor 2. At page 7, lines 8-23, the '054 application states that this C-terminal region in the Interleukin-1 receptor contains residues essential for IL-1R signaling. At page 7, lines 8-23 and in Example 11, the '054 application states that when this 13 amino acid C-terminal region in the Toll-like receptor 2 polypeptide is deleted, the resulting truncated variant can no longer induce NF-κB activation.

6. Prior to and at the time of the filing of the '054 application, I was working in the field of technology relating to Toll proteins at the DNAX Research Institute in Palo Alto, CA. I am a principal author of the article entitled "A Family of Human Receptors Structurally Related to *Drosophila* Toll" published in 1998 in the Proceedings of the National Academy of Sciences, Volume 85, pages 588-593. I note that this article was cited by Genentech in the '054 application. See, e.g. '054 application at page 2, line 25.

7. In the article referred to in Paragraph 6 above, I and my co-authors teach that given the sequence homology between the cytoplasmic domains of Toll polypeptides and the cytoplasmic domains of human interleukin 1 receptors, it is expected that both molecules trigger signaling pathways tied to Rel-type transcription factors such as NF-κB. See, e.g. page 588. The article further teaches that, as suggested from sequence homology data, Toll-like receptor 4 activates NF-κB and triggers the production of several inflammatory cytokines, hallmarks of an innate immune response. See the note at page 593 which describes the disclosure of Medzhitov et al., Nature 388, 394-397 (1997).

8. I believe that based on at least the description in the '054 application, the sequence homology data relating to PRO258, and the state of the art, one skilled in the art would reasonably understand that PRO285 can induce the activation of NF-κB and/or the expression of NF-κB-controlled genes and that antibodies to PRO285 could be made and used in accordance with routine techniques to modulate such activity.

9. I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: December 1, 2003

J. FERNANDO BAZAN, Ph.D.

DECLARATION
ATTACHMENT A

Curriculum Vitae

JOSE FERNANDO BAZAN, PH.D.

426 Waverley St. #6
Palo Alto, CA 94301-1727
USA

tel 650.321.9436
cell 650.224.0686
email jfbazan@proteinmachines.org
web www.proteinmachines.org

Personal

U.S. Citizen
Fluent in English and Spanish

Education

Ph.D. in Biophysics, December 1989
University of California, Berkeley

thesis Structural, evolutionary and crystallographic studies of growth factors, viral proteases and bifunctional enzymes (Advisors: Tom Jukes, UCB, and Robert Fletterick, UCSF)

B.S. in Physics, June 1982
Stanford University

thesis e+/e- collisions on the SPEAR ring at SLAC (Advisor: Alan Litke)

Professional

DNAX Research Institute (1993-2002)

Senior Staff Scientist, Dept. of Genomics (Mar. 1998-Nov. 2002)

Functional and comparative genomics, proteomics, molecular immunology, receptor mechanisms of signal transduction, receptor/ligand matching, protein structure & design, drug discovery & development, *in vivo* animal models, inflammatory and autoimmune diseases

Staff Scientist, Dept. of Molecular Biology (Dec. 1994-Feb. 1998)

Structural and computational biology, novel immune factor discovery, molecular recognition in receptor/ligand engagements, protein engineering, innate immune receptors (TLRs)

Visiting Fellow, Frank Lee lab, Dept. of Molecular Biology (Oct. 1993-Dec. 1994)

Cytokine structural biology, ADP Ribosyltransferases, novel factor discovery, protein structure prediction & modeling

Accomplishments

- Formed and led an innovative and very successful laboratory that was engaged in the discovery and characterization of novel proteins involved in critical immune regulatory pathways, weaving together state-of-the-art bioinformatics, biophysical and cellular methods with functional genomic and proteomic tools.

- Predicted an unusual enzymatic activity for CD38, confirmed by experiment. Sparked research into ADP-ribosyltransferases, finding a large family of human enzymes whose biological functions continue to be explored in collaboration with other investigators.
- Critically helped identify the cytokine ligand for the Flt-3 receptor by seeking a sequence fragment suggesting a predicted fold & receptor similiarity with stem cell factor and M-CSF. This has been recently confirmed by crystallographic studies.
- Played a leading role in the discovery, characterization and receptor matching of a number of novel IL-1-like, IL-17-like, TNF/C1Q-family, and hemopoietic (NNT-1, TSLP, IL-10-like, IL-23, IL-27, IL-28-like) cytokines.
- First to identify and clone the full complement of human and mouse Toll-like receptors (TLRs) that are now under intense study as critical innate immune receptors.
- Initiated efforts to develop novel molecules (such as TSLP, IL-23 and IL-27) and specific monoclonal antibodies as therapeutic entities for the treatment of a variety of human immune disorders.
- Guided the design and implementation (with Beth Basham) of web-accessible databases that catalogued and linked together in-house protein projects (sequences, cDNAs, structural & prediction data, antibodies, purified protein stock, etc.) with external sites (NCBI, PubMed, Interpro, various microarray data depositories) and tools (sequence comparison, fold recognition, transmembrane & signal peptide prediction, genomic).
- Pioneered (in collaboration with R. Kastelein & D. Gorman research groups) an integrated, systems-based approach to molecular analysis at DNAX. This multidisciplinary approach partly comprised:
  - (i) Utilizing sensitive computational & structure-based tools to discover fold relatives in broad genomic databases
  - (ii) Developing the facility to rapidly clone and characterize the relevant families of (putatively) expressed genes, and capturing their chromosomal loci for subsequent gene deletion projects
  - (iii) Using EST spread, microarray and Taqman-generated data to derive comparative expression profiles of genes of interest
  - (iv) Expressing proteins (or predicted domain fragments) in a variety of systems (bacterial, yeast, insect cell, mammalian cell) typically by retroviral or adenoviral infection
  - (v) Emphasizing biochemical tools (such as circular dichroism, calorimetry) to purify, analyze and confirm the predicted structures & activities of proteins
  - (vi) Using structural models for protein enginecrin, such as in the design of cytokine antagonists.
  - (vii) Speeding drug design by employing X-ray and NMR techniques to elucidate the structure of compelling molecules in collaborations with groups at Stanford and Berkeley,
  - (viii) Performing biophysical (like Biacore) and genetic studies to discern & catalogue the complex interactions of families of interacting ligands and receptors
  - (ix) Guiding novel proteins through an array of *in vitro* and *in vivo* experiments (from cell-based to animal disease models) to validate their use in clinical applications

Current research

- Novel hemopoietic cytokines that regulate the differentiation of naïve T cells into Th1 and Th2 developmental subtypes
- Mechanisms of innate *vs.* adaptive immunity—primitive defense factors and receptors that trigger cytokine pathways
- Utilizing comparative genomics approaches to map the emergence of the various superfamilies (organized by fold class) of immune regulators across the spectrum of completed genomes
- Designing a web site (linked to www.proteinmachines.org) to publish a complete structural taxonomy—from either determined or accurately modeled proteins—of cytokines and their receptors, with a focus on molecular recognition and signaling facets
- Capturing novel peptide and small molecule antagonists of cytokine receptor signaling by targeting novel fold intermediates
- Genetic basis and molecular mechanisms of complex human diseases (with particular interest in type I diabetes and other autoimmune & inflammatory disorders).

University of California, San Francisco (1985-1993)

Postdoctoral Fellow (Oct. 1991-Oct. 1993)
Structural biology: protein and drug design
Dept. of Biochemistry & Biophysics, Program of Excellence, Cardiovascular Research Institute
Robert J. Fletterick & Lewis T. Williams, Principal Investigators.

Postdoctoral Fellow, Oct. 1989-Oct. 1991
Molecular evolution of protein structure (Alfred P. Sloan Fellowship)
Department of Biochemistry & Biophysics
Robert J. Fletterick, Principal Investigator.

- Early involvement in the application of novel 2^0 and 3^0 structure prediction, fold recognition and modeling techniques (exploiting evolutionary information inherent in families of distant sequences) to successfully predict the structures and biochemical functions of proteins.
- Discovered and characterized a novel family of viral proteases essential for picorna & flavivirus maturation whose predicted folds and activities have been confirmed by X-ray studies and are active targets of drug design efforts.
- First defined & categorized the conserved structural architectures of various superfamilies of cytokines & their receptors (notably hemopoietic) with a focus on their modes of engagement, and mechanisms of specificity & signal transduction. These predictions have been validated by structural work, and laid the foundation for a broad genomics-based effort to identify novel molecules by fold recognition methods.
- Argued successfully that a published crystal structure of IL-2 was incorrectly traced, and modeled an alternative fold that agreed better with experimental data.
- Trained in experimental X-ray crystallography and biochemistry (Fletterick lab), evolutionary molecular biology (Jukes lab), computational biology (Fred Cohen lab), and immunology (Williams lab), the latter work introducing me to DNAX, a preeminent immunological institute.

Stanford University (1978-1982)

Research Assistant, 1987-89
Depts. of Pharm. Chemistry, and Biochemistry & Biophysics
Structural biology and targeted drug design for AIDS (NIH study)
George Kenyon, Director.

Research Assistant, 1987-88
Dept. of Neurology, School of Medicine
Prion structural biology (NIH Trainee)
Stanley Prusiner, Principal Investigator.

Research Technician, 1980-81
Senior thesis research: Experimental high-energy particle physics at the Stanford Linear Accelerator
Department of Physics, Stanford University
Alan Litke, Principal Invesigator.

Research Technician, 1979
Department of Physics, Stanford University
Stuart Freedman, Principal Investigator.

Teaching

Recurring lectures in Molecular Immunology, 2001-present
(Structure & Evolution of Cytokine Receptors; Signaling Mechanisms of Innate Immune Pathways)
Advanced Immunology 211/212 graduate courses, K.C. Garcia (organizer),
Stanford University

Teaching Assistant, 1983-1985
Physics 7A-C: Introductory physics for scientists &engineers (with laboratory)
Department of Physics, University of California, Berkeley
- Head T.A., 1983 (Oversaw the quality of teaching of over 20 T.A.'s, conducted large review lectures, organized exam and homework grading)

Teaching Assistant, 1980-82
Physics 51-58: Mechanics, electricity & magnetism and modern physics (with laboratory)
Department of Physics, Stanford University
- Head T.A., 1981 (Coordinated T.A.'s in lab and lecture duties, exam and paper grading)

Mentoring

Graduate students & Postdoctoral fellows (with Current Positions)

Jorge Guimarães, M.D., Ph.D., 1995-1997
(Faculty & clinical post at the Univ. of Oporto Medical School, Portugal)

Fernando L. Rock, Ph.D., 1994-1997
(Staff Scientist, PPD Discovery, Menlo Park)

Gary Hardiman, Ph.D., 1994-1997
(Director of the Biomedical Genomics Microarray Facility at UC, San Diego)

Sriram Balasubramanian, Ph.D., 1995-1996 (with Gerard Zurawski)
(Senior Staff Scientist at Axys Pharmaceuticals/Celera, South San Francisco)

Theo Sana, Ph.D., 1995-1999 (with Rob Kastelein)
(Staff Scientist, Agilent, Palo Alto)

Frederich Koch-Nolte, M.D., 1994, and 1997
(Faculty position at the University Clinic, Hamburg)

Birgit Oppmann, Ph.D., 1997-2000 (with Rob Kastelein)
(Max-Delbruck-Zentrum fur Molekulare Medizin, Berlin)

Reno Debets, Ph.D., 1997-2000 (with Rob Kastelein)
(Faculty position, Dept. of Medical Oncology, Erasmus Medical Center & the Daniel den Hoed Clinic, Rotterdam)

Beth Basham, Ph.D., 1998-2000
(Staff Scientist, DNAX Research Institute, Palo Alto)

Pedro Reche, Ph.D., 1998-2001
(Principal Investigator, Molecular Immunology Foundation, Dana Farber Cancer Institute, Harvard Medical School)

Stefan Pflanz, Ph.D., 1999-2002 (with Rob Kastelein)
(Staff Scientist, Micromet, Munich)

Joao Pereira, 2001-2002
(Ph.D. Graduate student, Pasteur Institute, Paris)

Peter Kirk, Ph.D., 2000-present

Jochen Schmitz, Ph.D., 2001-present

Alexander Owyang, Ph.D., 2002-present

Honors

Alfred P. Sloan Foundation Fellow in Studies of Molecular Evolution, 1990-91

University Fellowship, 1985-87, University of California, Berkeley.

Rebecca Carrington Award, June 1982, to a graduating senior in Physics, Stanford University.

President, Society of Physics Students, 1980-81, Stanford University.

Patents

Author on over 45 patent applications relating to the discovery and therapeutic application of novel molecules; a dozen of these have been granted in the U.S. as of Dec. 2002.

Societies

AAAI, AAAS, American Crystallographic Association, The Cytokine Society, The Protein Society

Publications

In preparation or submitted

Schmitz, J., **Bazan, J. F.** (2003) A long LOST cytokine is found. *Trends Immunol.* In preparation.

Kirk, P., **Bazan, J. F.** (2003) IL-17 receptors explained. *In preparation.*

Bazan, J. F., Kastelein, R. A. (2003) The control of Th1 development by the IL-27, IL-12 and IL-23 axis of cytokines. *Ann. Rev. Immunol.*, in preparation.

Bazan, J. F. (2003) Structure and evolution define the interactions and signaling mechanisms of hemopoietic cytokines and their receptors. *Adv. Prot. Chem.*, in preparation.

Reche, P., **Bazan, J. F.** (2003) Symmetry and function in death domain fold architecture. *J. Molec. Biol.*, in preparation.

Bazan, J. F. (2003) Origins of the hematopoietic cytokine helical fold. *J. Molec. Biol.*, in preparation.

Owyang, A., **Bazan, J. F.** (2002) An evolutionary intermediate in the divergence of IL-10 and IFN-α/β families of cytokines. *Trends Immunol.*, in preparation.

Bazan, J. F. (2002) Invertebrate helical cytokines. *Curr. Biol.*, submitted.

Bazan, J. F. (2002) Predicted membrane topology and globular architecture of Wolframin, a complex transmembrane protein implicated in Wolfram's Syndrome. *Proteins*, submitted.

Kirk, P., Pereira, J., **Bazan, J. F.** (2002) A genomic perspective on the TLR signaling pathway. *Genome Res.*, submitted.

Bazan, J. F. (2002) Receptor complexes: a paradigm revisted. *Nature Struct. Biol.*, submitted.

Boonstra, A., Crain, C., Liu, Y.-J., Pereira, J., Kastelein, R. A., **Bazan, J. F.**, de Waal-Malefyt, R., Vieira, P., O'Garra, A. (2002) Differential expression of Toll-like receptors on mouse dendritic cell subsets: expression profile and functional consequences. *J. Exp. Med.*, submitted.

Refereed journal articles

Hibbert, L., Pflanz, S., Vaisberg, E., Rosales, R., **Bazan, J. F.**, de Waal-Malefyt, R., Kastelein, R. A. (2002) IL-27 and IFNα induce T-bet and IL-12Rβ2 in naïve T-cells. *Nature Immunol.*, in press.

Bazan, J. F. (2002) Evolution of TLR signaling mechanisms. *Biochem. Soc. Trans.*, in press.

Glowacki G., Braren, R., Firner, K., Nissen, M., Kuhl, M., Reche, P., **Bazan, J. F.**, Cetkovic-Cvrlje, M., Leiter. E., Haag, F., Koch-Nolte, F. (2002) The family of toxin-related ecto-ADP-ribosyltransferases in humans and the mouse. *Protein Sci.* 11, 1657-1670.

Koch-Nolte, F., Reche, P., Haag, F., **Bazan, J. F.** (2001) ADP- ribosyltransferases: plastic tools for inactivating protein and small molecular weight targets. *J. Biotechnol.* 92, 81-87.

Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., **Bazan, J. F.**, de Waal-Malefyt, R., Rennick, D., Kastelein, R. A. (2002) IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* 16, 779-790.

Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gormand, D., Zurawski, S., Abrams, J., Menon, S., McClanahan, T., de Waal-Malefyt, R., **Bazan, J. F.**, Kastelein, R. A., Liu, Y.-J. (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nature Immun.* 3, 673-680.

Sims, J., Nicklin, M. J., **Bazan, J. F.**, Barton, J. L., Busfield, S. J., Ford, J. E., Kastelein, R. A., Kumar, S., Lin, H., Mulero, J., Pan, J. G., Pan, Y., Smith, D. E., Young, P. R. (2001) A new nomenclature for IL-1 family genes. *Trends Immunol. 22,* 536-537.

Kadowaki, N., Ho, S., Antonenko, S., de Waal-Malefyt, R., Kastelein, R. A., **Bazan, J. F.**, Liu, Y.-J. (2001) Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med 194,* 863-870.

Neighbors, M., Xu, X., Barrat, F. J., Ruuls, S. R., Churakova, T., Debets, R., **Bazan, J. F.**, Kastelein, R. A., Abrams, J. S., O'Garra, A. (2001) A critical role for interleukin 18 in primary and memory effector responses *to Listeria monocytogenes* that extends beyond its effects on interferon-γ production. *J. Exp. Med 194,* 343-354.

Reche, P. A., Soumelis, V., Gorman, D. M., Clifford, T., Liu, M.-R., Travis, M., Zurawski, S. M., Johnston, J., Liu, Y.-J., Spits, H., de Waal-Malefyt, R., Kastelein, R. A., **Bazan, J. F.** (2001) Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J. Immunol.* 167, 336-343.

Lougheed, J. C., Holton, J. M., Alber, T., **Bazan, J. F.**, Handel, T. M. (2001) Structure of melanoma inhibitory activity protein, a member of a recently identified family of secreted proteins. *Proc Natl Acad Sci U. S. A.* 98, 5515-20.

Fahrer, A. M., **Bazan, J. F.**, Papathanasiou, P., Nelms, K. A., Goodnow, C. C. (2001) A genomic view of immunology. *Nature* 409, 836-838.

Debets, R, Timans, J. C., Churakowa, T., Zurawski, S., de Waal-Malefyt, R., Moore, K. W., Abrams, J. S., O'Garra. A., **Bazan, J. F.**, Kastelein, R. A. (2000)IL-18 receptors, their role in ligand binding and function: anti-IL-1RAcPL antibody, a potent antagonist of IL-18. *J. Immunol.* 165, 4950-4956.

Taylor, K. R., Holzer, A. K., **Bazan, J. F.**, Walsh, C. A., Gleeson, J. G. (2000). Patient mutations in doublecortin define a repeated tubulin-binding domain. *J. Biol Chem.* 275, 34442-34450.

Sana, T. R., Debets, R., Timans, J. C., **Bazan, J. F.**, Kastelein, R. A. (2000) Computational identification, cloning, and characterization of IL-1R9, a novel interleukin-1 receptor-like gene encoded over an unusually large interval of human chromosome Xq22.2-q22.3. *Genomics* 15, 252-262.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M.-R., Gorman, D.M., Wagner, J., Zurawski, S., Liu, Y.-J., Abrams, J.S., Moore, K.W., de Waal-Malefyt, R., Hannum, C., Rennick, D., **Bazan, J. F.**, Kastelein, R.A. (2000) Novel p19 protein engages IL-12 p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13, 715-725.

Wiekowski, M., Leach, M., Evans, E., Sullivan, L, Chen, S.-C., **Bazan, J. F.**, Gorman, D.M., Kastelein, R.A., Narula, S. Lira, S.A. (2001) Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J. Immunol.* 166, 7563-7570.

Pan, S., Tsuruta, R., Masuda, E. S., Imamura, R., **Bazan, J. F.**, Arai, K., Arai, N., Miyatake, S. (2000) NFATz: a novel Rel similarity domain containing protein. *Biochem Biophys Res Commun.* 272, 765-776.

Debets, R., Timans, J. C., Homey, B., Zurawski, S., Sana, T. R., Lo, S., Wagner, J., Edwards, G., Clifford, T., Menon, S., **Bazan, J. F.**, Kastelein, R. A. (2001) Two novel IL-1 family members, IL-1δ and IL-1ε, function as an antagonist and agonist of NF-κB activation through the orphan IL-1R related protein 2. *J. Immunol.* 167, 1440-1446.

Patel, N., Brinkman-Van der Linden, E. C., Altmann, S. W., Gish, K., Balasubramanian, S., Timans, J. C., Peterson, D., Bell, M. P., **Bazan, J. F.**, Varki, A., Kastelein, R. A. (1999) OB-BP1/Siglec-6. a leptin- and sialic acid-binding protein of the immunoglobulin superfamily. *J. Biol. Chem.* 274, 22729-22738.

Rossi, D. L., Hardiman, G., Copeland, N. G., Gilbert, D. J., Jenkins, N., Zlotnik, A., **Bazan, J. F.** (1999) Cloning and characterization of a new type of mouse chemokine. *Genomics* 47, 163-170

Mizoue, L. S. , **Bazan, J. F.**, Handel, T. M. (1999). Solution structure and dynamics of the chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1. *Biochemistry* 38, 1402-1414.

Cacalano, N. A., Migone, T. S., **Bazan, J. F.**, Hanson, E. P., Chen, M., Candotti, F., O'Shea, J. J., Johnston, J. A. (1999) Autosomal SCID caused by a point mutation in the N-terminus of Jak3: mapping of the Jak3-receptor interaction domain. *EMBO J* 18, 1549-1558.

Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., **Bazan, J. F.** (1998). A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA* 95, 588-593.

Rossi, D., Hardiman, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Zlotnik, A., **Bazan, J. F.** (1997). Cloning and characterization of a new type of mouse chemokine. *Genomics* 47, 163-170.

Robinson, D., Shibuya, K., Mui, A., Zonin, F., Kastelein, R. A., **Bazan, J. F.**, O'Garra, A. (1997). IGIF does not drive Th1 development but synergizes with IL-12 for interferon-γ production and activates IRAK and NF-κB. *Immunity* 7, 571-581.

Bazan, J. F., Goodman, C. S. (1997). Modular structure of *Drosophila* Beat. *Curr. Biol.* 7, 338-339.

Hardiman, G., Kastelein, R. A., **Bazan, J. F.** (1997). Isolation, characterization and chromosomal location of human Wnt10B. *Cytogenet. Cell Genet.* 77, 278-282.

Guimarães, M. J., McClanahan, T., **Bazan, J. F.** (1997) Application of differential display in studying developmental processes. *Methods Mol Biol.* 85,175-194.

Hardiman, G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Kastelein, R.A., **Bazan, J. F.** (1997). Genetic structure and chromosomal mapping of *Myd88*. *Genomics* 45, 332-339.

Hunter, C. A., Timans, J. C., Piscane, P., Menon, S., Cai, G., Walker, W., Aste-Amezaga, M., Chizzonite, R., **Bazan, J. F.**, Kastelein, R. A. (1997). Comparison of the effects of interleukin-1α, interleukin-1β and interferon-γ-inducing factor on the production of interferon-γ by Natural Killer cells. *Eur. J. Immunol.* 27, 2787-2792

Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Rossi, D., Greaves, D. R., Zlotnik, A., Schall, T. J. (1997). A new class of membrane-bound chemokine with a CX_3C motif. *Nature* 385, 640-644.

Kim, I.-Y., Guimarães, M. J., Zlotnik, A., **Bazan, J. F.**, Stadtman, T. C. (1997). Fetal mouse selenophosphate synthase 2 (SPS2): characterization of the cysteine mutant form overproduced in a baculovirus insect cell system. *Proc. Natl. Acad. Sci. USA* 94, 418-421.

Hardiman, G., Rock, F. L., Balasubramanian, S., Kastelein, R. A., **Bazan, J. F.** (1996) Molecular characterization and modular analysis of human Myd88. *Oncogene* 13, 2467-2475.

Guimarães, M. J., Peterson, D., Vicari, A., Cocks, B. J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Ferrick, D. A., Kastelein, R. A., **Bazan, J. F.**, Zlotnik, A. (1996). Identification of a novel *selD* homolog from eukaryotes, bacteria and archaebacteria: Is there an autoregulatory mechanism in selenocysteine metabolism? *Proc. Natl. Acad. Sci. USA* 93, 15086-15091.

Rock, F. L., Peterson, D., Weig, B. C., Kastelein, R. A., **Bazan, J. F.** (1996). Binding of leptin to the soluble ectodomain of recombinant leptin receptor: a kinetic analysis by surface plasmon resonance. *Horm. Metab. Res.*, 28, 748-750.

Rock, F. L., Altmann, S. W., van Heek, M., Kastelein, R. A., **Bazan, J. F.** (1996). The leptin haemopoietic cytokine fold is stabilized by an intrachain disulfide bond. *Horm. Metab. Res.*, 28, 649-652.

Koch-Nolte, F., Haag, F., Braren, R., Kuh, M., Hoovers, J., Balasubramanian, S, **Bazan, J. F.**, Thiele, HG (1996) Two novel human members of an emerging mammalian gene family related to mono-ADP-ribosylating bacterial toxins. *Genomics* 39, 370-376.

Koch-Nolte, F., Haag, F., Kastelein, R. A., **Bazan, J. F.** (1996). Uncovered: the family relationship of a T-cell membrane protein and bacterial toxins. *Immunol. Today* 17, 402-405.

Guimarães, M. J., **Bazan, J. F.**, Castagnola, J., Diaz, S., Copeland, NG, Gilbert, D.J., Jenkins, N.A., Varki, A. Zlotnik, A. (1996). Molecular cloning and characterization of lysosomal sialic acid *O*-acetyl-transferase. *J. Biol. Chem.* 271, 13697-13705.

Koch-Nolte, F., Petersen, D., Balasubramanian, S., Haag, F., Kahlke, D., Willer, T., Kastelein, R. A., **Bazan, J. F.**, Thiele, HG (1996). Mouse T-cell membrane proteins RT6-1 and RT6-2 are arginine protein

mono(ADPRibosyl)-transferanses and share secondary structure motifs with ADP-ribosylating bacterial toxins. *J. Biol. Chem.* 271, 7686-7693.

Bazan, J. F., Schall, T. J. (1996). Interleukin-16 or not? *Nature* 381, 29-30.

Bazan, J. F. (1996). Big rigs in blood coagulation. *Nature* 380, 21-22.

Bazan, J. F., Timans, J. C., and Kastelein, R. A. (1996). A newly defined interleukin-1? *Nature* 379, 591.

Bazan, J. F. (1996). Helical fold prediction for the cyclin box. *Proteins* 24, 1-17.

Altmann, S. W., Timans, J. C., Rock, F.L., **Bazan, J. F.**, Kastelein, R. A. (1995) Expression and purification of a synthetic human obese gene product. *Protein Exp. Purif.* 6, 722-726.

Guimarães, M. J., **Bazan, J. F.**, Zlotnik, A., Wiles, M. V., Grimaldi, J. C., Lee, F., McClanahan, T. (1995). A new approach to the study of haemopoietic development in the yolk sac and embryoid bodies. *Development* 121, 3335-3346.

Fletterick, R. J., **Bazan, J. F.** (1995). When one and one are not two. *Nature Struct. Biol.* 2, 721-723.

Bazan, J. F. (1995). Postmodern complexes. *Nature* 376, 217-218.

Grimaldi, J. C., Balasubramanian, S., Kabra, N. H., Shanafelt, A., **Bazan, J. F.**, Zurawski, G., Howard, M. C. CD-38-mediated ribosylation of proteins. *J. Immunol.* 155, 811-817.

Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyenstuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., **Bazan, J. F.**, Moore, K. W., Schall, T. J., Zlotnik, A. (1994). Lymphotactin: a cytokine that represents a new class of chemokine. *Science* 266, 1395-1399.

Hannum, C., Culpepper, J., Campbell, D., McClanahan, T., Zurawski, S., **Bazan, J. F.**, Kastelein, R. A., Hudak, S., Wagner, J., Mattson, J., *et al.* (1994) Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. *Nature* 368, 643-648.

Bazan, J. F., Weaver, L. H., Roderick, S. L., Huber, R., Matthews, B. W. (1994). Sequence and structural comparison suggest that methionine aminopeptidase, prolidase, aminopeptidase P and creatinase share a common fold. *Proc. Natl. Acad. Sci. USA*, 91. 2473-2477.

Rozwarski, D., **Bazan, J. F.**, Clore, G. M., Bohm, A., Hatada, M., Wlodawer, A., Karplus, P. A. (1993). Structural comparisons among the short chain helical cytokines. *Structure* 2, 159-173.

Ho, A. S., Liu, Y., Khan, T. A., Hsu, D. H., **Bazan, J. F.**, Moore, K. W. (1996). A receptor for interleukin-10 is related to interferon receptors. *Proc. Natl. Acad. Sci. USA* 90, 11267-11271.

Banchereau, J., **Bazan, J. F.**, Blanchard, D., Briere, F., Galizzi, J. P., van Kooten, C., Liu, Y. J., Rousset, F., Saeland, S. (1994) The CD40 antigen and its ligand. *Ann. Rev. Immunol.* 12, 881-922.

Sprang, S. R., **Bazan, J. F.** (1993) Cytokine structural taxonomy and mechanisms of receptor engagement. *Curr. Opin. Struc. Biol.* 3, 815-827.

Bazan, J. F. (1993) Emerging families of cytokines and receptors. *Curr. Biol.* 3, 603-606.

Howard, M., Grimaldi, J. C., **Bazan, J. F.**, Lund, F. E., Santos-Argumedo, L., Parkhouse, R. M. E., Walseth, T., Lee, H. C. (1993) Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* 262, 1056-1059.

Wolfsberg, T. G., **Bazan, J. F.**, Blobel, C. P., Myles, D. G., Primakoff, P., and White, J. M. (1993) The precursor region of a protein active in sperm-egg fusion contains a metalloprotease and a disintegrin domain: structural, functional and evolutionary implications. *Proc. Natl. Acad. Sci. USA* 90, 10783-10787.

Rawlings, D. J., Saffran, D. C., Tsukuda, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., **Bazan, J. F.**, Howard, M., Copeland, N. G., Jenkins, N. A., and Witte, O. N. (1993) Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 261, 358-361.

Bazan, J. F. (1992) Unraveling the structure of interleukin-2. *Science* 257, 410-413.

Sander, C., Vriend, G., **Bazan, J. F.**, Horovitz, A., *et al.* (1992) Protein design on computers - 5 new proteins - Shpilka, Grendel, Fingerclasp, Leather and Aida. *Proteins* 12, 105-110.

Gautier, J. P., Solomon, M., Booher, R., **Bazan, J. F.**, Kirschner, M. (1991) Cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell* 67, 197-211.

Bazan, J. F. (1991) WKS motifs and the cytokine receptor framework of tissue factor. *Trends Biochem. Sci.* 16, 329.

Bazan, J. F. (1991) Neuropoietic cytokines in the hematopoietic fold. *Neuron* 7, 197-208.

Bazan, J. F. (1991) Genetic and structural homology of stem cell factor and macrophage colony-stimulating factor. *Cell* 65, 9-10.

Bazan, J. F., Fletterick, R. J. (1990) Structural and catalytic models of trypsin-like viral proteases. *Semin. Virol.* 1, 311-322.

Chambers, T. J., Weir, R. C., Grakoui, A., McCourt, D. W., **Bazan, J. F.**, Fletterick, R. J., Rice, C. M. (1990) Evidence that the N-terminal domain of yellow fever virus NS3 is a serine protease responsible for site- specific cleavages in the viral polyprotein. *Proc. Natl. Acad. Sci. USA* 87, 8898-8902.

Bazan, J. F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* 87, 6934-6938.

Bazan, J. F. (1990) Haemopoietic receptors and helical cytokines. *Immun. Today* 11, 350-354.

Bazan, J. F. (1990) Shared architecture of hormone-binding domains in type I and type II interferon receptors. *Cell* 61, 753-754.

Bazan, J. F. (1989) A novel family of growth factor receptors: A common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor β-chain. *Biochem. Biophys. Res. Commun.* 164, 788-795.

Bazan, J. F., Fletterick, R. J., Pilkis, S. J. (1989) Evolution of a bifunctional enzyme: 6-Phosphosfructo-2-kinase/Fructose-2,6-bisphosphatase. *Proc. Natl. Acad. Sci. USA* 86, 9642-9646.

Dougherty, W. G., Parks, T. D., Cary, S. M., **Bazan, J. F.**, Fletterick, R. J. (1989) Characterization of the catalytic residues of the tobacco etch virus 49-kDa proteinase. *Virology* 172, 302-310.

Bazan, J. F., Fletterick, R.J. (1989) Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. *Virology* 171, 637-639.

Bazan, J. F., Fletterick, R. J. (1989) Comparative analysis of viral cysteine protease structural models. *FEBS Lett.* 249, 5-7.

Bazan, J. F., Fletterick, R. J. (1988) Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: Structural and functional implications. *Proc. Natl. Acad. Sci. USA* 85, 7872-7876.

Braun, M. J., Gonda, M. A. , George, D. G., **Bazan, J. F.**, Fletterick, R. J., and Prusiner, S. B. (1987) The burden of proof in linking AIDS to scrapie. *Nature* 330, 525-526.

Bazan, J. F., Fletterick, R. J., and Prusiner, S. B. (1987) AIDS virus and scrapie protein genes. *Nature* 325, 581.

Pastuszyn, A., Noland, B. J., **Bazan, J. F.**, Fletterick, R. J., and Scallen, T. J. (1987) Primary sequence and structural analysis of sterol carrier protein from rat liver. *J. Biol. Chem.* 262, 13219-13227.

Bazan, J. F., Fletterick, R. J., McKinley, M. P., and Prusiner, S. B. (1987) Predicted secondary structure and membrane topology of the scrapie prion protein. *Protein Engin.* 1, 125-135.

Book chapters

Braren, R., Firner, K., Balasubramanian, S., **Bazan, J. F.**, Thiele, H. G., Haag, F., Koch-Nolte, F. (1997). Use of the EST database resource to identify and clone novel mono(ADP-ribosyl)transferase gene family members. In *ADP-ribosylation in animal tissues: Structure, function and biology of mono (ADP-ribosyl) transferases and related enzymes*, Haag, F., and Koch-Nolte, F., Eds. (Plenum Press, New York), pp. 163-8.

Bazan, J. F., Koch-Nolte, F. (1997). Sequence and structural links between distant ADP-ribosyltransferase families. *ibid*, pp. 99-107.

Wilkens, K, Tiemann, B., **Bazan, J. F.**, Ruger, W. (1997). ADP-ribosylation and early transcription regulation by bacteriophage T4. *ibid*, pp. 71-82.

Guimarães, M. J., McClanahan, T., **Bazan, J. F.** (1996) Application of differential display in studying developmental processes, in *Differential display by PCR and related techniques for gene discovery*, Pardee, A., and Liang, P., Eds. (Cold Spring Harbor Lab. Press).

Bazan, J. F., Fletterick, R. J. (1990) Tertiary structure modeling of 6-phosphosfructo-2-kinase/fructose-2,6-bisphosphatase: Evolutionary, structural and functional design of a bifunctional enzyme, in *Fructose-2-6-Bisphosphate*, Pilkis, S. J. , Ed. (CRC Press, Boca Raton, Fl.), pp. 125-171.

Bazan, J. F. (1989) Viral branches of the trypsin-like proteinase family, in *Viral proteinases as targets for chemotherapy*, Krausslich, H.-G., Oroszlan, S. and Wimmer, E., Eds. (Cold Spring Harbor Lab. Press), pp. 55-62.

Bazan, J. F., Horowitz, A., Nakamura, H., and Ribas, L. (1991) Design and analysis of novel handshake structures, in *Protein Design on Computers 1990, EMBL Biocomputing Tech. Doc.* 6, Sander, C. and Vriend, G., Eds. (EMBL, Heidelberg), pp. 105-132.

Finer-Moore, J. , **Bazan, J. F.**, Rubin, J., Stroud, R. M. (1988) Identification of membrane proteins and soluble protein secondary structural elements, domain structure and packing arrangements by fourier-transform amphipathic analysis, in *Prediction of protein structure and the principles of protein conformation*, G. Fasman, Ed. (Plenum Press, New York), pp. 719-759.

Conference Proceedings

Bazan, J. F., Rock, F. L., Hardiman, G., Timans, J. C., and Kastelein, R. A. (1997). A novel family of human receptors structurally related to *Drosophila* Toll–Evolutionary links between patterning and immunity in vertebrates and insects. In *Pattern formation during development, LXII Cold Spr. Harb. Symp. Quant. Biol.*, Stillman, B., Ed. (Cold Spr. Harb. Press, NY), p. 23 (Abstract).

Cohen, F. E. C., Gregoret, L., Kuntz, I. D., **Bazan, J. F.** (1988) Theoretical studies of protein folding, in A.A.A.S. seminar: The protein folding problem, Boston, Feb. 12-14.

Others

Oppmann, B., Zhong, J., Xu, Y-M., Timans, J. C., Han, J., de Waal-Malefyt, R., Hannum, C., Rose-John, S., Patterson, P. H., **Bazan, J. F.**, Kastelein, R. (2000). Novel Neurotrophin-1 (NNT-1) and soluble receptor NR6 form a composite cytokine that engages the CNTF signaling complex. *Unpublished.*

Guimarães, M. J., Hudak, S., Vicari, A., Rossi, D., Leach, M., Hill, K. E., Burk, R. F., Pina-Cabral, J. M., Rennick, D., **Bazan, J. F.** (1997) Thymic atrophy and delayed kinetics of hematopoietic repopulation in selenium deficient mice. *Unpublished.*

Bazan, J. F. (1989) *Ph.D. Thesis*. University of California, Berkeley. Structural, evolutionary and crystallographic studies of growth factors, viral proteases and bifunctional enzymes.

Bazan, J. F., Cohen, F. E. C. (1987) Structure and evolution of related lymphokines, hemopoetins and growth hormones. *Unpublished monograph.*

Talks

Cold Spring Harbor meeting on *Viral Proteinases as Targets for Chemotherapy*, May 1989 (invited speaker).

Int. Soc. Interferon Res. Annual Meeting in San Francisco, Nov. 1990 (invited speaker).

165th Ciba Foundation meeting on Multifunctional Cytokines, Oct. 1991 (invited speaker).

13th Int. Conf. on Biochemical Analysis, Growth Factors Symposium, Munich, May 1992 (invited speaker).

PENCE meeting on Cytokine structure, Vancouver, Jan. 1993 (invited speaker).

Keystone symposium on Cytokines: From clone to clinic. Keystone, Jan., 1993 (workshop organizer).

NY Acad. of Sciences meeting on Primordial Immunity, Woods Hole, Mar. 1993 (invited speaker).

52nd Pittsburg Diffraction Conference, Pennsylvania, 1994 (invited speaker).

International meeting of the Cytokine Society, Lake Tahoe, October 1994 (invited speaker, session organizer).

Cytokine and receptor structures, ASBMB meeting, Anaheim, 1994 (workshop organizer).

Novel cytokines and receptors, AAI meeting, San Francisco, 1995 (workshop organizer).

ASMB-BMB meeting, San Francisco, 1995 (invited speaker; session organizer).

1st international workshop on ADP-ribosyltransferases, Hamburg, May 1996 (plenary speaker).

Joint International meeting of the Cytokine Society, and the Society for Interferon and Cytokine Research, Geneva, October 1996 (plenary speaker).

National Institutes of Health, Cytokine Signaling Symposium, March 1997 (plenary speaker).

FASEB/AAAI meeting, San Francisco, April 1998 (invited speaker).

Perspectives in Cytokine Research, British Cytokine Group, London, UK, June 1998 (plenary speaker).

2nd Joint Meeting of the International Cytokine Society & the Int. Soc. For Interferon and Cytokine Research, Jerusalem, Oct. 1998 (plenary speaker).

Hematopoietic Cytokine Symposium in Mainz,Germany, March 1999 (plenary speaker).

2nd International Aachen Workshop on Cytokine Signaling, Aachen, Germany, March 2000 (invited speaker).

International Congress of Immunology 2001, Stockholm, Sweden, July 2001 (workshop chair).

Le Croisic meeting of the French Immunology society, May 2002(plenary speaker).

3rd International Aachen Workshop on Cytokine Signaling, Aachen, Germany, October 2002 (invited speaker).

Joint Meeting of the International Cytokine Society & the Int. Soc. for Interferon and Cytokine Research, Turin, Italy, October 2002 (plenary speaker).

Biochemical Society Focused Meeting on Toll-like Receptors, Horsham, U.K., Feb. 2003 (invited speaker).

Specificity and Pathophysiology of Signal Transduction Pathways meeting, Christian-Albrechts-Universität, Kiel, Germany, March 2003 (invited speaker).

Cytokines from Structure to Therapy, Symposium of the Danish Biochemical Society, Copenhagen, Denmark, April 2003 (invited speaker).

Academic and biotech seminars

Affymax Research Institute; Amgen; Arris Pharmaceuticals; Cal Tech; Dartmouth Medical School; EMBL; Genentech; Gilead Sciences; Immunex; Incyte Pharmaceuticals; Millennium Pharmaceuticals; Protein Design Labs; Rutgers University Center for Advanced Biotechnology and Medicine, Schering-Plough Research Institute; Scios; Stanford Medical School, Dept. of Immunology; SUNY Stony Brook, Dept. of Physiology and Biophysics; Vertex Pharmaceuticals.

Workshops

Invited participant at an intensive workshop on Computer-assisted Protein Design, EMBL, Heidelberg, Sept.-Oct. 1990.

Invited participant in five CASP (Critical Assessment of techniques in protein structure prediction) workshops, Asilomar, 1994-2002.

Reviewer

Ad hoc review of manuscripts for *Protein Science, Protein Engineering, Proteins, Structure, Cell, Immunity, J. Immunology, Nature, Nature Immunology, Nature Structural Biology, Science, Neuron, Biochemistry, PNAS, Current Biology* and *Trends* journals

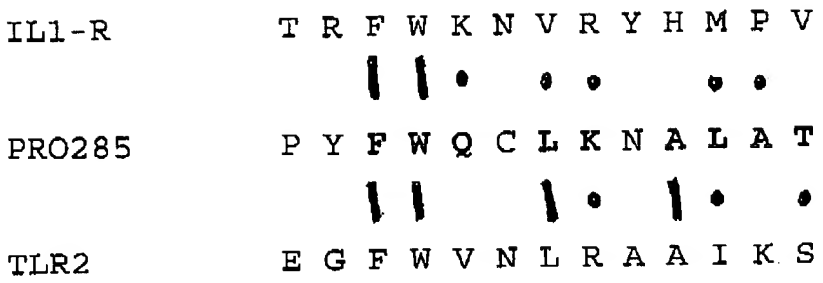
Interests

Soccer, mountain biking, hiking, travel; Latin American cuisine, music & culture

References

Available upon request

DECLARATION
ATTACHMENT B



• Indicates amino acids having conserved side chain properties.

EXHIBIT B

nature immunology

Volume 3 No 6 - June 2002

Correspondence

Nature Immunology 3, 499 (2002)
doi:10.1038/ni0602-499

Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848

Marion Jurk[1, 3], Florian Heil[2, 3], Jörg Vollmer[1], Christian Schetter[1], Arthur M. Krieg[1], Hermann Wagner[2], Grayson Lipford[1] & Stefan Bauer[2]

[1] Coley Pharmaceutical GmbH, Elisabeth-Selbert-Strasse 9, 40764 Langenfeld, Germany and Coley Pharmaceutical Group, Inc., 93 Worcester St., Wellesley, MA 02481, USA.

[2] Institute for Medical Microbiology, Hygiene and Immunology, Trogerstrasse 9, 81675 Munich, Germany.

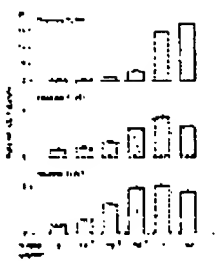
[3] These authors contributed equally to this work.

Correspondence should be addressed to S Bauer stefan.bauer@lrz.tum.de

Toll-like receptors (TLRs) play an important role in the innate immune response to pathogens[1]. TLRs detect PAMPs (pathogen-associated molecular patterns) and stimulate immune cells *via* the MyD88-dependent interleukin 1 receptor (IL-1R)–TLR signaling pathway, which leads to activation of the transcription factor NF-κB[2]. In humans, ten members of this family (TLR1 to TLR10) have been reported to date. TLR2, TLR4 and TLR5 are crucial for the recognition of peptidoglycan, lipopolysaccharide and flagellin, respectively[3-5]. TLR6 associates with TLR2 and recognizes lipoproteins from mycoplasma[6]. TLR9 detects bacterial DNA containing unmethylated CpG motifs and TLR3 activates immune cells in response to double-stranded RNA[7-9]. The natural ligands for TLR1, TLR7, TLR8 and TLR10 are not known, although a synthetic compound with antiviral activity has now been described as a ligand for TLR7[10].

Hemmi *et al.* reported in *Nature Immunology* that, in experiments that used gene-deficient mice, the antiviral imidazoquinoline resiquimod (R848) activates immune cells *via* the TLR7 MyD88-dependent signaling pathway[10]. They showed that macrophages from MyD88- and TLR7-deficient mice do not respond to R848 stimulation, whereas macrophages from wild-type mice strongly induce the transcription factor NF-κB and the secretion of proinflammatory cytokines, such as tumor necrosis factor-α, as well as the regulatory cytokine IL-12. In addition, Hemmi *et al.* showed, by genetic complementation in HEK293 cells, that human TLR7 confers responsiveness to R848[10].

In the quest to identify ligands for TLRs, we also screened immunostimulatory synthetic compounds for their potential to activate HEK293 cells that were transiently transfected with TLR cDNAs and a NF-κB luciferase reporter plasmid. We found that R848 induced NF-κB activation in HEK293 cells transfected with human TLR8 (GenBank accession number AF245703) in a dose-dependent manner (Fig. 1). In accordance with the findings of Hemmi *et al.*, human and murine TLR7 (GenBank accession numbers AF240467 and AY035889) also mediate R848 recognition (Fig. 1)[10]. TLR7 showed a higher sensitivity to R-848, but TLR8 was able to induce NF-κB more effectively than TLR7 when higher concentrations of R-848 were used (Fig. 1). In contrast, HEK293 cells transiently transfected with murine TLR8 (GenBank accession number AY035890) did not activate NF-κB after stimulation with R848 (data not shown), which suggests that TLR8 is nonfunctional in mice. This mute phenotype is in accordance with the observation that TLR7-deficient mice do not respond to R848, even though TLR8 is present[10].



High resolution image and legend (10K)

Figure 1. R-848 induces NF-κB activation via TLR8 or TLR7.

HEK293 cells were transfected by electroporation with vectors expressing human TLR8, human TLR7 or murine TLR7 and a NF-kB luciferase reporter plasmid. Sixteen hours after transfection, cells were stimulated with R848 (commercially synthesized by GLSynthesis, Worcester, MA) at the indicated concentrations for a further 7 h. TLR2-, TLR3- and TLR4-transfected cells did not induce NF-kB activation after stimulation with R848; a negative result was also scored for murine TLR8 (data not shown). Data are mean ± s.d. from one representative of three independent experiments.

These results show that both human TLR7 and TLR8 can independently mediate recognition of the same antiviral compound, imidazoquinoline R848, which suggests a possible redundancy among these receptors. The differential dose response observed between these receptors may allow fine-tuning of the immune response to high or low concentrations of this molecule as well as other ligands. Further studies will be necessary to identify the natural ligands for both receptors and clarify the status and function of murine TLR8.

References

1. Medzhitov, R. *Nature Rev. Immunol.* **1**, 135-145 (2001). | Article | PubMed |
2. Medzhitov, R. *et al. Mol. Cell* **2**, 253-258 (1998). | PubMed | ISI |
3. Takeuchi, O. *et al. Immunity* **11**, 443-451 (1999). | PubMed | ISI |
4. Poltorak, A. *et al. Science* **282**, 2085-2088 (1998). | Article | PubMed | ISI |
5. Hayashi, F. *et al. Nature* **410**, 1099-1103 (2001). | Article | PubMed | ISI |
6. Ozinsky, A. *et al. Proc. Natl. Acad. Sci. USA* **97**, 13766-13771 (2000). | Article | PubMed | ISI |
7. Hemmi, H. *et al. Nature* **408**, 740-745 (2000). | Article | PubMed | ISI |
8. Bauer, S. *et al. Proc. Natl. Acad. Sci. USA* **98**, 9237-9242 (2001). | Article | PubMed | ISI |
9. Alexopoulou, L., Holt, H.C., Medzhitov, R. & Flavell, R.A. *Nature* **413**, 732-738 (2001). | Article | PubMed | ISI |
10. Hemmi, H. *et al. Nature Immunol.* **3**, 196-200 (2002). | Article | PubMed | ISI |

EXHIBIT C

groove-type sites may be derived from the same family of germline V_H genes bearing the QUPC 52 idiotype (18).

The large number of environmental carbohydrate antigens and the high degree of specificity of antibodies elicited in response to each carbohydrate antigen suggest that a tremendous diversity of antibody molecules must be available, from which some antibodies can be selected for every possible antigenic structure. In order to regulate such a diverse system, a network theory has been proposed, in which antibodies are themselves recognized as antigenic (see Chapters 12 and 24, and ref. 19), and the response to streptococcal polysaccharide is a leading example in which anti-idiotypic antibodies can be shown to regulate the response to antigen (20).

Recent studies of a series of 17 monoclonal anti-α (1 → 6) dextran hybridomas (21,22) have investigated whether the binding sites of closely related antibodies would be derived from a small number of variable region genes, for both heavy and light chains, or whether antibodies of the same specificity could derive from variable region genes with highly divergent sequences. Each monoclonal had a groove-type site that could hold six or seven sugar residues (with one exception), based on inhibition of immunoprecipitation by different length oligosaccharides. Thus, unlike monoclonals to haptenated proteins, the precise epitope could be well characterized and was generally quite similar among the entire series.

Studies of the V_κ sequences revealed that only three V_κ groups were used in these hybridomas. Use of each V_κ group correlated with the particular antigen used to immunize the animals, whether linear dextran or short oligosaccharides, so that 10 of the monoclonals from mice immunized the same way all used the same V_κ.

In contrast, the 17 V_H chains were derived from at least five different germline genes from three different V_H gene families (23). The two most frequently used germline V_H genes were found in seven and five monoclonals, respectively, with minor variations explainable by somatic mutations. Once again, V_H gene usage correlated with size of the antigen used to immunize, although the length of each CDR did not correlate with the size of the groove-type binding site. The remarkable finding is that very different V_H chains (about 50% homologous) can combine with the same V_K to produce antibody-binding sites with nearly the same size, shape, antigen specificity, and affinity. A similar phenomenon can also occur when different V_H sequences combine with different V_K sequences to produce antibodies with very similar properties. This is a result of the fact that dextran binding depends on the antigen fitting into the groove and interacting favorably with the residues forming the sides and bottom of the groove. The results indicate that divergent variable region sequences, both in and out of the complementarity-determining regions, can be folded to form similar binding site contours, which result in similar immunochemical characteristics. Similar results have been reported in other antigen–antibody systems, such as phenyloxazolone (24).

More recently, these studies were expanded to include 34 groove-type monoclonal anti-α(1 → 6) dextran-binding hybridomas (25), of which 10 used heavy chain V_H19.1.2 and eleven used V_H9.14.7. Starting with different V_H genes, these two families of monoclonals provide an experiment of nature concerning the ability of each V_H gene to combine with different light chain V_K and J_K genes, as well as heavy chain D and J_H genes to produce a groove-shaped binding site of a given specificity. In every one of these 21 monoclonals, the same light chain V_K-Ox1 gene was used, but the V_H19 family used a single J_K sequence exclusively (J_K2), while the V_H9 family included all four of the active J_K segments (J_K1, 2, 4, and 5). Similarly, the heavy chain J_H sequences of the V_H19 family were all of a single type (J_H3), while those of the V_H9 family included three types (J_H1, 2, and 3). A single D region was used by both families (DFL16.1), but the junctional sequences between V_H–D and D–J_H were different, with the V_H19 using minimal substitutions, and the V_H9 allowing more variability in junctional sequences, depending on the size of the J_H with which it was joining. Although the amino acid sequences of these two V_H genes are 73% identical, they use markedly different strategies to arrive at the same groove-type binding site with nearly identical size and specificity. The results suggest that the two heavy chain variable regions, perhaps due to their conformation, may place diff rent structural constraints on which minigene components can successfully contribute to forming a particular site. Two different strategies for generating antibody specificity are apparent, even though the same V_K and D minigenes were used by both families. For the V_H19 family, point mutations in the CDR2 generated the α(1 → 6) dextran specificity, while the rest of the structure was held constant. For the V_H9 family, a wide variety of J_H, J_K, and V_H–D and D–J_H sequences were used to generate the groove-type site. These two blueprints for constructing a binding site may also reflect distinct cellular pathways for generating antibody diversity.

Protein and Polypeptide Antigenic Determinants

Like the proteins themselves, the antigen determinants of proteins consist of amino acid residues in a particular three-dimensional array. The residues that make contact with complementary residues in the antibody-combining site are called contact residues. To make contact, of course, these residues must be exposed on the surface of the protein, not buried in the hydrophobic core. Since the complementarity-determining residues in the hypervariable regions of antibodies have been found to span as much as 30 to 40 Å × 15 to 20 Å × 10 Å (D. R. Davies, *personal communication*), these contact

residues comprising the antigenic determinant may cover a significant area of protein surface, as now measured in a few cases by x-ray crystallography of antibody–protein antigen complexes (26–28). Looked at from another point of view, the size of the combining sites has been estimated using simple synthetic oligopeptides of increasing length, such as oligolysine. In this case, a series of elegant studies (29–31) suggested that the maximum length of chain a combining site could accommodate was six to eight residues, corresponding closely to that found earlier for oligosaccharides (14,15), discussed previously.

Several types of interactions contribute to the binding energy. Many of the amino acid residues exposed to solvent on the surface of a protein antigen will be hydrophilic. These are likely to interact with antibody contact residues via polar interactions. For instance, an anionic glutamic acid carboxyl group may bind to a complementary cationic lysine amino group on the antibody, or vice versa; or a glutamine amide side chain may form a hydrogen bond with the antibody. However, hydrophobic interactions can also play a major role. Proteins cannot exist in aqueous solution as stable monomers with too many hydrophobic residues on their surface. Those hydrophobic residues that are on the surface can contribute to binding to antibody for exactly the same reason. When a hydrophobic residue in a protein antigenic determinant or, similarly, in a carbohydrate determinant (8) interacts with a corresponding hydrophobic residue in the antibody-combining site, the water molecules previously in contact with each of them are excluded. The result is a significant stabilization of the interaction. A thorough review of these aspects of the chemistry of antigen–antibody binding has recently been published (32).

Mapping Epitopes: Conformation Versus Sequence

The other component that defines a protein antigenic determinant, besides the amino acid residues involved, is the way these residues are arrayed in three dimensions. Since the residues are on the surface of a protein, we can also think of this component as the topography of the antigenic determinant. Sela (33) divided protein antigenic determinants into two categories, sequential and conformational, depending on whether the primary sequence or the three-dimensional conformation appeared to contribute the most to binding. On the other hand, since the antibody-combining site has a preferred topography in the native antibody, it would seem *a priori* that some conformations of a particular polypeptide sequence would produce a better fit than others and therefore would be energetically favored in binding. Thus conformation or topography must always play some role in the structure of an antigenic determinant.

Moreover, when one looks at the surface of a protein in a space-filling model, one cannot ascertain the direction of the backbone or the positions of the helices (contrast Figs. 3 and 4). It is hard to recognize whether two residues that are side by side on the surface are adjacent on the polypeptide backbone or whether they come from different parts of the sequence and are brought together by the folding of the molecule. If a protein maintains its native conformation when an antibody binds, then it must similarly be hard for the antibody to discriminate between residues that are covalently connected directly and those connected only through a great deal of intervening polypeptide. Thus the probability that an antigenic determinant on a native globular protein consists of only a consecutive sequence of amino acids in the primary structure is likely to be rather small. Even if most of the determinant were a continuous sequence, other nearby residues would probably play a role as well. Only if the protein were cleaved into fragments before the antibodies were made would there be any reason to favor connected sequences.

This concept was analyzed and confirmed quantitatively by Barlow et al. (39), who examined the atoms lying within spheres of different radii from a given surface atom on a protein. As the radius increases, the probability that all the atoms within the sphere will be from the same continuous segment of protein sequence decreases rapidly. Correspondingly, the fraction of surface atoms that would be located at the center of a sphere containing only residues from the same continuous segment falls dramatically as the radius of the sphere increases. For instance, for lysozyme, with a radius of 8 Å, fewer than 10% of the surface residues would lie in such a "continuous patch" of surface. These are primarily in regions that protrude from the surface. With a radius of 10 Å, almost none of the surface residues fall in the center of a continuous patch. Thus for a contact area of about 20 Å × 25 Å, as found for a lysozyme–antibody complex studied by x-ray crystallography, none of the antigenic sites could be completely continuous segmental sites.

Antigenic sites consisting of amino acid residues that are widely separated in the primary protein sequence but brought together on the surface of the protein by the way it folds in its native conformation have been called "assembled topographic" sites (40,41) because they are assembled from different parts of the sequence and exist only in the surface topography of the native molecule. By contrast, the sites that consist of only a single continuous segment of protein sequence have been called "segmental" antigenic sites (40,41).

In contrast to T cell recognition of "processed" fragments retaining only primary and secondary structures, the evidence is overwhelming that most antibodies are made against the native conformation when the native protein is used as immunogen. For instance, antibodies to nativ staphylococcal nuclease were found to have about a 5000-fold higher affinity for the native protein

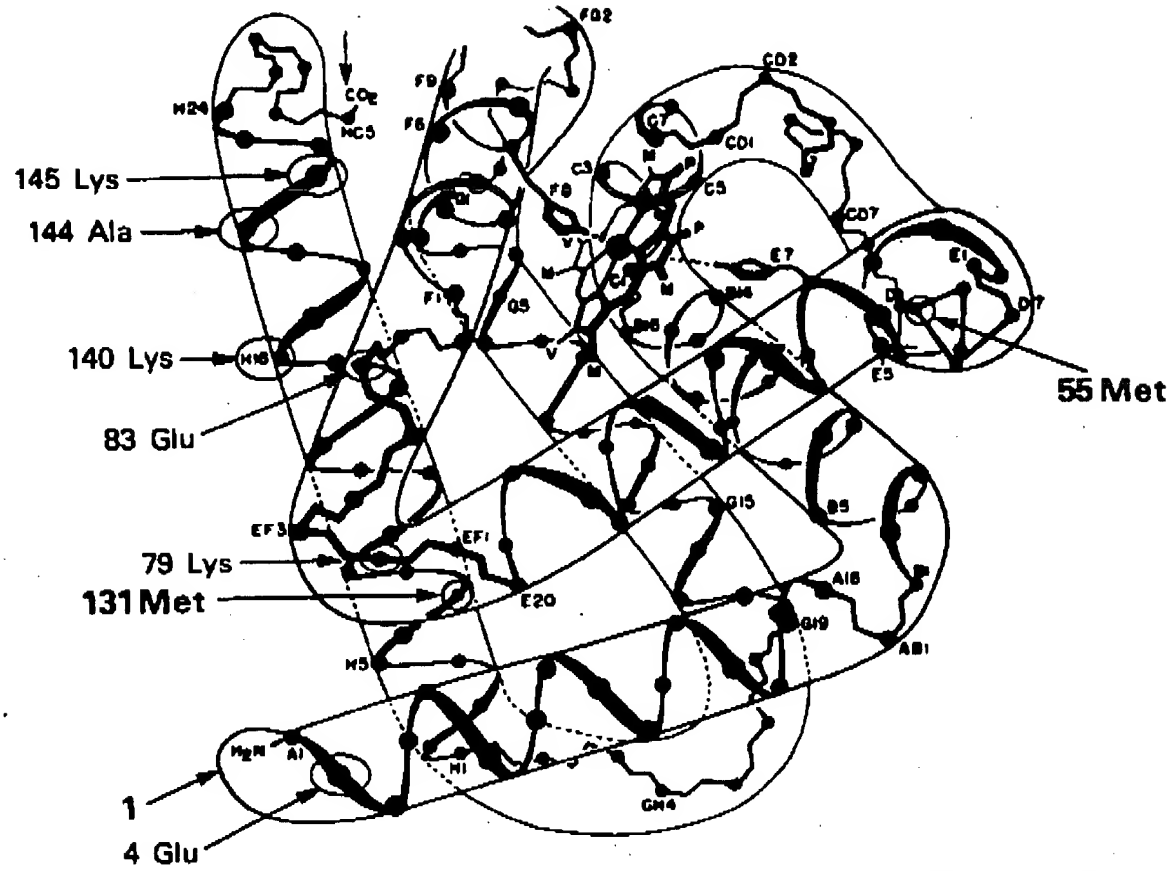


FIG. 3. Artist's representation of the polypeptide backbone of sperm whale myoglobin in its native three-dimensional conformation. The α helices are labeled A through H from the amino terminal to the carboxyl terminal. Side chains are omitted, except for the two histidine rings (F8 and E7) involved with the heme iron. Methionines at positions 55 and 131 are the sites of cleavage by cyanogen bromide (CNBr), allowing myoglobin to be cleaved into three fragments. Most of the helicity and other features of the native conformation are lost when the molecule is cleaved. A less drastic change in conformation is produced by removal of the heme to form apomyoglobin, since the heme interacts with several helices and stabilizes their positions relative to one another. The other labeled residues (4 Glu, 79 Lys, 83 Glu, 140 Lys, 144 Ala, and 145 Lys) are residues that have been found to be involved in antigenic determinants recognized by monoclonal antibodies (34). Note that cleavage by CNBr separates Lys 79 from Gly^{34} 4 and separates Glu 83 from Ala 144 and Lys 145. The "sequential" determinant of Koketsu and Atassi (35) (residues 15 to 22) is located at the elbow, **lower right**, from the end of the A helix to the beginning of the B helix. (Adapted from ref. 36.)

than for the corresponding polypeptide on which they were isolated (by binding to the peptide attached to Sepharose) (42). An even more dramatic example is that demonstrated by Crumpton (43) for antibodies to native myoglobin or to apomyoglobin. Antibodies to native ferric myoglobin produced a brown precipitate with myoglobin, an indication that the heme was still in the protein in what was, at least approximately, its native environment. Such antibodies did not bind well to the apomyoglobin, which, without the heme, has a slightly altered conformation. On the other hand, antibodies to the apomyoglobin, when mixed with native (brown) myoglobin, produced a white precipitate. These antibodies so strongly favored the conformation of apomyoglobin, from which the heme was excluded, that they trapped those molecules that vibrated toward that conformation and pulled the equilibrium state over to the apo form. One could almost say, figuratively, that the antibodies squeezed the heme out of the myoglobin. Looked at thermodynamically, it is clear that the conformational preference of the antibody for the apo versus native forms, in terms of free energy, had to be greater than the free energy of binding of the heme to myoglobin. Thus, in general, antibodies are made that are very specific for the conformation of the protein used as immunogen.

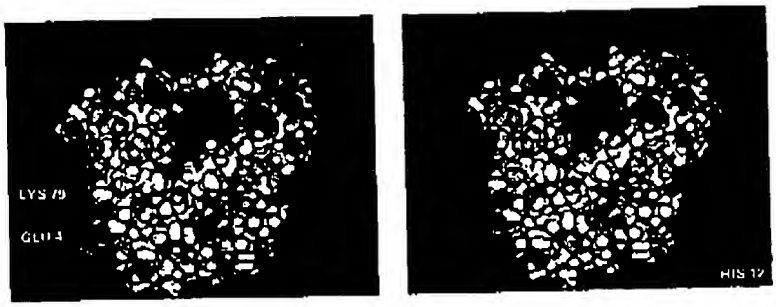


FIG. 4. Stereoscopic views of a computer-generated space-filling molecular model of sperm whale myoglobin, based on the Takano (37) x-ray diffraction coordinates. This orientation, which corresponds to that in Fig. 3, is arbitrarily designated the "front view." The computer method was described by Feldmann et al. (38). The heme and aromatic carbons are *shaded darkest*, followed by carboxyl oxygens, then other oxygens, then primary amino groups, then other nitrogens, and finally side chains of aliphatic residues. The backbone and the side chains of nonaliphatic residues, except for the functional groups, are shown in *white*. Note that the direction of the helices is not apparent on the surface, in contrast to the backbone drawing in Fig. 3. The residues Glu 4, Lys 79, and His 12 are believed to be part of a topographic antigenic determinant recognized by a monoclonal antibody to myoglobin (34). This stereo pair can be viewed in three dimensions using an inexpensive stereoviewer such as the "stereoscopes" sold by Abrams Instrument Corp., Lansing, MI, or Hubbard Scientific Co., Northbrook, IL. (Adapted from ref. 34.)

A number of methods have been used to identify the antigenic determinants bound by particular antibodies made against a protein. Binding to cleavage fragments and short synthetic peptides from the protein sequence has been the most widely used approach. The synthetic peptides may be made by conventional solid-phase peptide synthesis (44) or by methods designed to make large numbers of peptides for screening. In one such method, multiple peptides are made simultaneously in separate polypropylene mesh "tea-bags" that can be put through the common steps in the sequence together and separated only for the different amino acid coupling steps (45). In another method, the peptides are synthesized on the tips of plastic pins inserted in the wells of 96-well microtiter plates in such a way that these can then be used for solid-phase binding assays of antibodies without ever cleaving the peptide off the plastic support (46). These two methods especially lend themselves to studying multiple variants of the natural sequence to identify the residues critical for antibody binding. Usually, the longer the peptides, the more that specificity can be confidently determined, as short peptides of only six to eight amino acid residues often manifest nonspecific binding (47). If the synthetic peptides correspond to segments of the protein antigen sequence, as is most common, then the use of peptides is limited to identifying the structures bound by antibodies specific for segmental antigenic sites.

To identify assembled topographic sites, more complex approaches have been necessary. The earliest was the use of natural variants of the protein antigen with known amino acid substitutions, where such evolutionary variants exist (40). Thus substitution of different amino acids in proteins in the native conformation can be examined. The use of this method, which is illustrated later, is limited to studying the function of amino acids that vary among homologous proteins, that is, those that are polymorphic. It may now be extended to other residues by use of site-directed mutagenesis. A second method is to use the antibody that binds to the native protein to protect the antigenic site from modification (48) or proteolytic degradation (49). A related but less sensitive approach makes use of competition with other antibodies (50–52). A third approach, taking advantage of the capability of producing thousands of peptides on a solid-phase surface for direct binding assays (46), is to study binding of a monoclonal antibody to every possible combination of six amino acids (46). If the assembled topographic site can be mimicked by a combination of six amino acids not corresponding to any continuous segment of the protein sequence but structurally resembling a part of the surface, then one can produce a "mimotope" defining the specificity of that antibody (46).

Myoglobin also serves as a good model protein antigen for studying the range of variation of antigenic determinants from those that are more sequential in nature to those that do not even exist without the native conformation of the protein (Fig. 3). A good example of the first, more segmental type of determinant is that consisting of residues 15 to 22 in the amino terminal portion of the molecule. Crumpton and Wilkinson (53) first discovered that the chymotryptic cleavage fragment consisting of residues 15 to 29 had antigenic activity for antibodies raised to either native or apomyoglobin. Synthetic peptides corresponding to the shorter sequence 15 to 22 were then found by two groups (35,54) to bind antibod-

ies made to native sperm whale myoglobin, even though the synthetic peptides were only 7 to 8 residues long. Peptides of this length do not spend much time (in solution) in a conformation corresponding to that of the native protein. On the other hand, these synthetic peptides had a several hundred-fold lower affinity for the antibodies than did the native protein. Thus, even if most of the determinant was included in the consecutive sequence 15 to 22, the antibodies were still much more specific for the native conformation of this sequence than for the random conformation peptide. Moreover, there was no evidence to exclude the participation of other residues, nearby on the surface of myoglobin but not in this sequence, in the antigenic determinant.[1]

A good example of the importance of secondary structure is the case of the loop peptide (residues 64 to 80) of hen egg-white lysozyme (59). This loop in the protein sequence is created by the disulfide linkage between cysteine residues 64 and 80 and has been shown to be a major antigenic determinant for antibodies to lysozyme (59). The isolated peptide 60 to 83, containing the loop, binds antibodies with high affinity, but opening of the loop by cleavage of the disulfide bond destroys most of the antigenic activity for antilysozyme antibodies (59).

At the other end of the range of conformational requirements are those determinants involving residues far apart in the primary sequences that are brought close together on the surface of the native molecule by its folding in three dimensions. Myoglobin also provides a good example of these determinants, which are called assembled topographic determinants (40,41). Of six monoclonal antibodies to sperm whale myoglobin studied by Berzofsky et al. (34,60), none bound to any of the three cyanogen bromide cleavage fragments of myoglobin that together span the whole sequence of the molecule. Therefore these monoclonal antibodies (all with affinities between 2×10^8 and 2×10^9 M^{-1}) were all highly specific for the native conformation. These were studied by comparing the relative affinities for a series of native myoglobins from different species with the known amino acid sequences of these myoglobins. With the myoglobins available, this approach allowed the definition of some of the residues involved in binding to three of these antibodies. The striking result was that two of these three monoclonal antibodies were found to recognize topographic determinants, as defined previously. One recognized a determinant including Glu 4 and Lys 79, which are on the A helix and E-F corner of the myoglobin molecule but come within about 2 Å of each other to form a salt bridge in the native molecule (Fig. 4). The other antibody recognized a determinant involving Glu 83 in the E-F corner, and Ala 144 and Lys 145 on the H helix of the myoglobin molecule (Fig. 5). Again, these are far apart in the primary sequence but are brought within 12 Å of each other by the folding of the molecule in its native conformation. Similar examples have recently been reported for monoclonal antibodies to human myoglobin (61) and to lysozyme (50). Other examples of such conformation-dependent antigenic determinants have been suggested using conventional antisera to such proteins as insulin (62), hemoglobin (63), tobacco mosaic virus (64), and cytochrome *c* (65). Moreover, the crystallographic structures of lysozyme–antibody (26,28) and neuraminidase–antibody (27) complexes show clearly that, in both cases, the epitope bound is an assembled topographic site.

How frequent are antibodies specific for topographic determinants compared to those that bind consecutive sequences when conventional antisera are examined? This question was studied by Lando et al. (66), who passed goat, sheep, and rabbit antisera to sperm whale myoglobin over columns of Sepharose-coupled cyanogen bromide cleavage fragments of myoglobin, together spanning the whole sequence. The antisera were passed sequentially, and repeatedly, over each of the three columns until no more antibodies could be removed. Nevertheless, 30% to 40% of the antibodies originally present in each serum remained after this treatment. These antibodies still bound to the native myoglobin molecule with high affinity but did not bind to any of the fragments in solution by radioimmunoassay. Thus, in four of four antimyoglobin sera tested, 60% to 70% of the antibodies could bind peptides and 30% to 40% could bind only native-conformation intact protein.

On the basis of studies such as these, it has been suggested that much of the surface of a protein molecule may be antigenic (40,67) but that the surface can be divided up into antigenic domains (34,57,58,61). Each of these domains consists of many overlapping determinants recognized by different antibodies.

An additional interesting point can be made from the above studies about the topography of protein antigenic determinants. If one examines the topographic determinant consisting of sperm whale myoglobin residues 83, 144, and 145, shown in stereo in Fig. 5, it is apparent that they are on both lips of a deep crevice or concavity in the protein surface. It is possible, although not yet demonstrated, that a complementary protuberance in the antibody-combining site actually inserts into this cavity. From the studies of myeloma proteins that bind small haptens or carbohydrates, we are accustomed to think of the antigen being engulfed by a cavity or crevice

[1] This is the only segmental antigenic determinant of myoglobin that has clearly been confirmed by more than one independent group of investigators. Crumpton and Wilkinson (53) did measure antigenic activity for a chymotryptic fragment 147 to 153 that overlaps one of the other reported sequential determinants (55). However, two of the other reported sequential determinants (55), corresponding to residues 56 to 62 and 94 to 100, have not been reproducible when tested with other antisera, even raised in the same species (56). For related studies, see refs. 57 and 58.

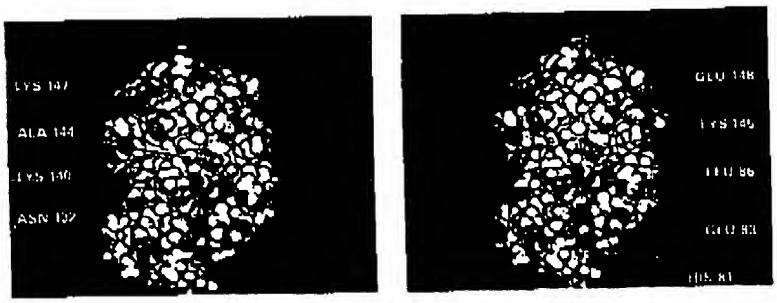


FIG. 5. Stereoscopic view of computer-generated space-filling model of the left view of sperm whale myoglobin, turned 90° relative to the view in Fig. 4. Methods and shading are as indicated in Fig. 4. The residues Glu 83, Ala 144, and Lys 145 are believed to be part of a topographic antigenic determinant recognized by a second monoclonal antibody to myoglobin (34). Note the concavity in the surface of the molecule in the middle of this determinant, between Glu 83 and Ala 144/Lys 145. (Adapted from ref. 34.)

on the antibody (68). However, for globular protein antigens binding to globular protein antibodies, the situation is more structurally symmetrical (and antigen–antibody binding is also thermodynamically symmetrical). Thus it is just as possible for a convexity on the antibody to insert into a concavity on the antigen as it is for the more conventional model to occur of a convexity on the antigen inserting into a concavity on the antibody. Now that monoclonal antibodies specific for protein antigens are available, we may encounter both types of cases. The determinant depicted in Fig. 5 might be such a case. In the three published crystal structures of protein antigen–antibody complexes, the contact surfaces were broad, with local complementary pairs of concave and convex regions in both directions (26–28). However, when we limit ourselves to antigenic sites defined with short peptides, which tend to identify sites that protrude from the surface of the antigen (39,69), we are likely to see a bias toward situations in which the antigen is convex and the antibody surface concave.

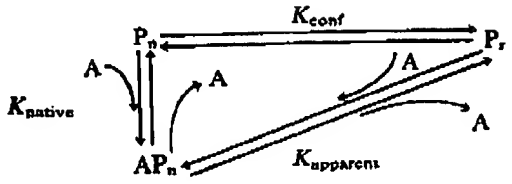
Further information on the subjects discussed in this section is available in the reviews by Sela (33), Crumpton (43), Reichlin (70), Kabat (68), Benjamin et al. (40), Berzofsky (41), and Getzoff et al. (32).

Conformational Equilibria of Protein and Peptide Antigenic Determinants

We have already referred to the fact that antibodies to a native protein have higher affinity for the native conformation than for other conformations of fragments or denatured molecules. Similarly, antibodies raised against fragments or denatured molecules generally have higher affinities for these forms than for the native conformation. In this section we discuss possible mechanisms for these affinity differences and explore how these can be used to advantage to study the conformational equilibria of proteins and peptides.

There are several possible mechanisms to explain why an antibody specific for a native protein will bind a peptide fragment in random conformation with lower affinity. Of course, the peptide may not contain all the contact residues of the antigenic determinant, so that the binding energy would be lower. However, for cases in which all the residues in the determinant are present in the peptide, several mechanisms still remain. First, the affinity may be lower because the topography of the residues in the peptide may not produce as complementary a fit in the antibody-combining site as the native conformation would. Second, it is possible that the apparent affinity is reduced because only a small fraction of the peptide molecules are in a nativelike conformation at any time. This model assumes that the antibody binds only those peptide molecules that are in the native conformation. Since the concentration of these is lower than the total peptide concentration by a factor that corresponds to the conformational equilibrium constant of the peptide, the apparent affinity is also lower by this factor. This model is analogous to an allosteric model. A third, intermediate hypothesis would suggest that initial binding of the peptide in a nonnative conformation occurs with submaximal complementarity and is followed by an intramolecular conformational change in the peptide to achieve energy minimization by assuming a nativelike conformation. This third hypothesis corresponds to an induced fit model. The loss of affinity is due to the energy required to change the conformation of the peptide, which in turn corresponds to the conformational equilibrium constant in the second hypothesis. To some extent these models could be distinguished kinetically, since the first hypothesis predicts a faster "on" rate and a faster "off" rate than does the second hypothesis (71).

Although not the only way to explain the data, the second hypothesis is useful because it provides a method to estimate the conformational equilibria of proteins and peptides (42,72). The method assumes the second hypothesis, which can be expressed as follows:



(1)

where A is antibody, P_n is native peptide, and P_r is random conformation peptide, so that

$$K_{apparent} = K_{conf}K_{native} \quad (2)$$

Thus the ratio of the apparent association constant for peptide to the measured association constant for the native molecule should give the conformational equilibrium constant of the peptide. Note the implicit assumption that the total peptide concentration can be approximated by $[P_r]$. This will generally be true, since most peptide fragments of proteins demonstrate little native conformation; that is, $K_{conf} = [P_n]/[P_r]$ is much less than 1. Also note that if the first hypothesis (or third) occurs to some extent, this method will overestimate K_{conf}. On the other hand, if the affinity for the peptide is lower because it lacks some of the contact residues of the determinant, this method will underestimate K_{conf} (by assuming that all the affinity difference is due to conformation). To some extent, the two errors may partially cancel out. When this method was used to determine the K_{conf} for a peptide staphylococcal nuclease, a value of 2×10^{-4} was obtained (42). Similarly, when antibodies raised to a peptide fragment were used, it was possible to estimate the fraction of time the native nuclease spends in nonnative conformations (72). In this case, the K_{conf} was found to be about 3000-fold in favor of the native conformation.

Antipeptide Antibodies that Bind to Native Proteins at a Specific Site

In light of the conformational differences between native proteins and peptides and the observed K_{conf} effects shown by antibodies to native proteins when tested on the corresponding peptides, it was somewhat surprising to find that antibodies to synthetic peptides show extensive cross-reactions with native proteins (73,74). These two types of cross-reactions can be thought of as working in opposite directions: the binding of antiprotein antibodies to the peptide is inefficient, while the binding of antipeptide antibodies to the protein is quite efficient and commonly observed. This finding is quite useful, since automated solid-phase peptide synthesis has become readily available. This has been particularly useful in three areas: exploitation of protein sequences deduced by recombinant DNA methods, preparation of site specific antibodies, and the attempt to focus the immune response on a single protein site that is biologically important but may not be particularly immunogenic. This section focuses on the explanation of the cross-reaction, uses of the cross-reaction, and the potential limitations with regard to immunogenicity.

The basic assumption is that antibodies raised against peptides in an unfolded structure will bind the corresponding site on proteins folded into the native structure (74). This is not immediately obvious, since antibody binding to antigen is the direct result of the antigen fitting into the binding site. Affinity is the direct consequence of "goodness of fit" between antibody and antigen, while antibody specificity is due to the inability of other antigens to occupy the same site. How then can the antipeptide antibodies overcome the effect of K_{conf} and still bind native proteins with good affinity and specificity? The whole process depends on the antibody-binding site forming a three-dimensional space and the antigen filling it in an energetically favorable way.

Since the peptides are randomly folded, they rarely occupy the native conformation, so they are not likely to elicit antibodies against a conformation they do not maintain. If the antibodies are specific for a denatured structure, then, like the myoglobin molecules that were denatured to apomyoglobin by antibody binding (43), the cross-reaction may depend on the native protein's ability to assume different conformational states. If the native protein is quite rigid, then the possibility of its assuming a random conformation is quite small; but if it is a flexible three-dimensional spring, then local unfolding and refolding may occur all the time. Local unfolding of protein segments may permit the immunologic cross-reaction with antipeptide antibodies, since a flexible segment could assume many of the same conformations as the randomly folded peptide (74).

In contrast, the proteins' ability to crystallize (a feature that allows the study of their structure by x-ray crystallography) has long been taken as evidence of protein rigidity (75). In addition, the existence of discrete functional states of allosteric enzymes (76) provides additional evidence of stable structural states of a protein. Finally, the fact that antibodies can distinguish native from denatured forms of intact proteins is well known for proteins such as myoglobin (43).

However, protein crystals are a somewhat artificial situation, since the formation of the crystal lattice imposes order on the components, each of which occupies a local energy minimum at the expense of considerable loss of randomness (entropy). Thus the crystal structure may have artificial rigidity that exceeds the actual rigidity of protein molecules in solution. On the contrary, we may attribute some of the considerable difficulty in crystallizing proteins to disorder within the native conformation. Second, allosterism may be explained by two distinct conformations that are discrete without being particularly rigid. Finally, the ability to generate antiprotein antibodies that are conformation specific does not rule out

the existence of antipeptide antibodies that are not. All antibodies are probably specific for some conformation of the antigen, but this need not be the crystallographic native conformation in order to achieve a significant affinity for those proteins or protein segments that have a "loose" native conformation.

Antipeptide antibodies have proved to be very powerful reagents when combined with recombinant DNA methods of gene sequencing (74,77). From the DNA sequence, the protein sequence is predicted. A synthetic peptide is constructed, coupled to a suitable carrier molecule, and used to immunize animals. The resulting polyclonal antibodies can be detected with a peptide-coated ELISA plate (see Chapter 12). They are used to immunoprecipitate the native protein from a ^{35}S-labeled cell lysate and thus confirm expression of the gene product in these cells. The antipeptide antibodies can also be used to isolate the previously unidentified gene product of a new gene. The site specific antibodies are also useful in detecting post-translational processing, since they bind all precursors and products that contain the site. In addition, since the antibodies bind only to the site corresponding to the peptide, they are useful in probing structure–function relationships. They can be used to block the binding of a substrate to an enzyme or the binding of a virus to its cellular receptor.

Immunogenicity of Proteins and Peptides

Up to this point, we have considered the ability of antibodies to react with proteins or peptides as antigens. However, immunogenicity refers to the ability of these compounds to elicit antibodies following immunization. In principle, nearly the entire surface of a globular protein should be able to elicit antibodies, particularly when we allow for both topographic and segmental specific antibodies (40). However, several factors limit the immunogenicity of different regions of proteins, and these have been divided into those that are intrinsic to protein structure itself versus those extrinsic to the antigen that are related to the responder and vary from one animal or species to another (41). In addition, we consider the special case of peptide immunogenicity as it applies to vaccine development.

Studies of intrinsic factors began by immunizing animals with native proteins and analyzing the antibodies that resulted. With polyclonal antisera, it is necessary to compare the relative amounts of antibodies directed at each site or class of sites: immunodominant sites are the ones that elicit the most antibodies. Monoclonal antibodies advanced our ability to study the immunogenicity of discrete sites. The features of protein structure that have been suggested to explain the results include surface accessibility of the site, hydrophilicity, flexibility, and proximity to a site recognized by helper T cells.

When the x-ray crystallographic structure and antigenic structure are known for the same protein, it is not surprising to find that a series of monoclonal antibodies binding to a molecule such as influenza neuraminidase choose an overlapping pattern of sites at the exposed head of the protein (78). The stalk of neuraminidase was not immunogenic, apparently because it was almost entirely covered by carbohydrate.

Beyond such things as carbohydrate, which may sterically interfere with antibody binding to protein, accessibility on the surface is clearly a *sine qua non* for an antigenic determinant to be bound by an antibody specific for the native conformation, without any requirement for unfolding of the structure (41). Several measures of such accessibility have been suggested. All these require knowledge of the x-ray crystallographic three-dimensional structure. Some have measured accessibility to solvent by rolling a sphere with the radius of a water molecule over the surface of a protein (79,80). Others have suggested that accessibility to water is not the best measure of accessibility to antibody and have demonstrated a better correlation by rolling a sphere with the radius of an antibody-combining domain (81). Another approach to predicting antigenic sites on the basis of accessibility is to examine the degree of protrusion from the surface of the protein (69). This was done by modeling the body of the protein as an ellipsoid and examining which amino acid residues remain outside ellipsoids of increasing dimensions. The most protruding residues were found to be part of antigenic sites bound by antibodies, but usually these sites had been identified by using short synthetic peptides and so were segmental in nature. As noted above, for an antigenic site to be contained completely within a single continuous segment of protein sequence, the site is likely to have to protrude from the surface, as otherwise residues from other parts of the sequence would fall within the area contacting the antibody (39).

Because the three-dimensional structure of most proteins is not known, other ways of predicting surface exposure have been proposed for the vast majority of antigens. For example, hydrophilic sites tend to be found on the water-exposed surface of proteins and could be favored targets for antibodies. Thus hydrophilicity has been proposed as a second indication of immunogenicity (82–84). This model has been used to analyze 12 proteins with known antigenic sites: the most hydrophilic site of each protein was indeed one of the antigenic sites. However, among the limitations are the facts that a significant fraction of surface residues can be nonpolar (79,80) and that several important examples of hydrophobic and aromatic amino acids involved in the antigenic sites are known (33,64,85,86). Specificity of antibody binding likely depends on the complementarity of surfaces for hydrogen bonding and polar bonding as well as van der Waals contacts (87), while hydrophobic interactions and

the exclusion of water from the interacting surfaces of proteins may contribute a large but nonspecific component to the energy of binding (87).

A third factor suggested to play a role in immunogenicity of protein epitopes is mobility. Measurement of mobility in the native protein is largely dependent on the availability of a high-resolution crystal structure, so its applicability is limited to only a small subset of proteins. Furthermore, it has been studied only for antibodies specific for segmental antigenic sites; therefore it may not apply to the large fraction of antibodies to assembled topographic sites. Studies of mobility have taken two directions. The case of antipeptide antibodies has already been discussed, in which antibodies made to peptides corresponding to more mobile segments of the native protein were more likely to bind to the native protein (74,88). This is not considered just a consequence of the fact that more mobile segments are likely to be those on the surface and therefore more exposed, because in the case of myohemerythrin (which was used as a model), two regions of the native protein that were equally exposed but less mobile did not bind nearly as well to the corresponding antipeptide antibodies (89). However, as is clear from the earlier discussion, this result applies to antibodies made against short peptides and therefore is not directly relevant to immunogenicity of parts of the native protein. Rather, it concerns the cross-reactivity of antipeptide antibodies with the native protein and therefore is of considerable practical importance for the purposes outlined in the section on antipeptide antibodies.

Studies in the other direction—that is, of antibodies raised against native proteins—would be by definition more relevant to the question of immunogenicity of parts of the native protein. Westhof et al. (90) used a series of hexapeptides to determine the specificity of antibodies raised against native tobacco mosaic virus protein and found that six of the seven peptides that bound antibodies to native protein corresponded to peaks of high mobility in the native protein. The correlation was better than could be accounted for just by accessibility, because three peptides that corresponded to exposed regions of only average mobility did not bind antibodies to the native protein. However, when longer peptides—on the order of 20 amino acid residues—were used as probes, it was found that antibodies were present in the same antisera that bound to less mobile regions of the protein (91). They simply had not been detected with the short hexapeptides with less conformational stability. Thus it was not that the more mobile regions were necessarily more immunogenic but rather that antibodies to these were more easily detected with short peptides as probes. A similar good correlation of antigenic sites with mobile regions of the native protein in the case of myoglobin (90) may also be attributed to the fact that seven of the nine sites were defined with short peptides of six to eight residues (55). Again, this result becomes a statement about cross-reactivity between peptides and native protein rather than about the immunogenicity of the native protein. For recent reviews, see Van Regenmortel (92) and Getzoff et al. (32).

To address the role of mobility in immunogenicity, an attempt was made to quantitate the relative fraction of antibodies specific for different sites on the antigen myohemerythrin (93). The premise was that, although the entire surface of the protein may be immunogenic, certain regions may elicit significantly more antibodies than others and therefore may be considered immunodominant or at least more immunogenic. Since this study was done with short synthetic peptides from 6 to 14 residues long based on the protein sequence, it was limited to the subset of antibodies specific for segmental antigenic sites. Among these, it was clear that the most immunogenic sites were in regions of the surface that were most mobile, convex in shape, and often of negative electrostatic potential. The role of these parameters has been covered in a recent review (32).

These results have important practical and theoretical implications. First, to use peptides to fractionate anti-protein antisera by affinity chromatography, peptides corresponding to more mobile segments of the native protein should be chosen when possible. If the crystal structure is not known, it may be possible to use peptides from amino or carboxyl termini or from exon–intron boundaries, as these are more likely to be mobile (88). Second, these results may explain how a large but finite repertoire of antibody-producing B cells can respond to any antigen in nature or even artificial antigens never encountered in nature. Protein segments that are more flexible may be able to bind by induced fit in an antibody-combining site that is not perfectly complementary to the average native structure (32,41). Indeed, evidence from the crystal structure of antigen–antibody complexes (94–96) suggests that mobility in the antibody-combining site as well as in the antigen may allow both reactants to adopt more complementary conformations on binding to each other, that is, a two-way induced fit. A very nice example comes from the study of antibodies to myohemerythrin (95), in which the data suggested that initial binding of exposed side chains of the antigen to the antibody promoted local displacements that allowed exposure and binding of other, previously buried residues. The role of these critical amino acid side chains that are buried in the native crystal structure appeared to be one of contact residues with the antibody-combining site rather than one of stabilization of a particular conformation. The only way this could occur would be for such residues to become exposed during the course of an induced fit conformational change in the antigen (32,95). In a second very clear example of induced fit, the contribution of antibody mobility to peptide binding was demonstrated for a monoclonal antibody to peptide 75–110 of influenza

h magglutinin, which was crystallized with or without peptide in the binding site and analyzed by x-ray crystallography for evidence of an induced fit (96). Despit flexibility of the peptide, the antibody-binding site probably could not accommodate the peptide without a conformational change in the third complementarity determining region (CDR3) of the heavy chain, in which an asparagine residue of the antibody was rotated out of the way to allow a tyrosine residue of the peptide to fit in the binding pocket of the antibody (96).

With regard to host-limited factors, immunogenicity is certainly limited by self-tolerance. Thus the repertoire of potential antigenic sites on mammalian protein antigens such as myoglobin or cytochrome *c* can be thought of as greatly simplified by the sharing of numerous amino acids with the endogenous myoglobin or cytochrome of the host. For mouse, guanaco, or horse cytochrome *c* injected into rabbits, each of the differences between the immunogen and rabbit cytochrome *c* is seen as an immunogenic site on a background of immunologically silent residues (40,65,97). In another example, antibodies to beef myoglobin were made in various species (98). Rabbit and dog antibodies bound almost equally well to beef or sheep myoglobin. However, sheep antibodies bound beef but not sheep myoglobin, even though these two myoglobins differ by just six amino acids. Thus the sheep immune system was able to screen out those clones that would be autoreactive with sheep myoglobin.

Ir genes of the host also play an important role in regulating the ability of an individual to make antibodies to a specific antigen (99). These antigen specific immunoregulatory genes are among the MHC genes that code for transplantation antigens. Structural mutations, gene transfer experiments, and biochemical studies (99) all indicate that *Ir* genes are actually the structural genes for MHC antigens. The mechanism of action of the MHC antigens works through their effect on helper T cells (described later). Briefly, T cells can respond to a protein antigen only when a fragment of the protein is bound to an MHC antigen, forming a compound antigen. T cells must be activated before they can help B cells respond to antigen by both expanding the appropriate clones of antigen specific B cells and differentiating the expanded clones into antibody-secreting plasma cells. There appear to be constraints on which B cells a T cell of a given specificity can help (100,101), a process called T–B reciprocity (102). Thus if *Ir* genes control helper T cell specificity, they will in turn limit which B cells are activated and thus which antibodies are made.

The immunogenicity of peptide antigens is also limited by intrinsic and extrinsic factors. Intrinsic features such as hydrophilicity and surface accessibility are not a problem, until we consider the cross-reaction of the elicited antibodies on the native protein. Bigger problems are the host-related factors extrinsic to the structure of the peptide. With less structure to go on, each small peptide must presumably contain some nonself structural feature in order to overcome self-tolerance. In addition, the same peptide must contain antigenic sites that can be recognized by helper T cells as well as by B cells. When no T cell site is present, three approaches may be helpful: graft on a T cell site; couple the peptide to a carrier protein; or overcome T cell nonresponsiveness to the available structure with various immunologic agents, such as interleukin 2.

An example of a biologically relevant but poorly immunogenic peptide is the asparagine–alanine–asparagine–proline (NANP) repeat unit of the circumsporozoite (CS) protein of malaria sporozoites. Studies with malaria parasites have shown that infection by the sporozoite form produced in the mosquito can be blocked by prior immunization with irradiated, killed sporozoites (103). A monoclonal antibody to CS protein can mimic the effect in murine malaria, and this antibody is specific for the repeat unit of the CS protein (104). Thus it would be desirable to make a malaria vaccine of the repeat unit of *P. falciparum* $(NANP)_n$. However, only mice of one MHC type ($H\text{-}2^b$) of all mouse strains tested were able to respond to $(NANP)_n$ (105,106). One approach to overcome this limitation is to couple $(NANP)_n$ to a site recognizable by T cells, perhaps a carrier protein such as tetanus toxoid (107). In human trials, this conjugate was weakly immunogenic and only partially protective. Moreover, as helper T cells produced by this approach are specific for the unrelated carrier, a secondary or memory response would not be expected to be elicited by the pathogen itself.

Another choice might be to identify a T cell site on the CS protein itself and couple the two synthetic peptides together to make one complete immunogen. The result with one such site, called Th2R, was to increase the range of responding mouse MHC types by one, to include $H\text{-}2^k$ as well as $H\text{-}2^b$ (108). This approach has the potential advantage of inducing a state of immunity that could be boosted by natural exposure to the sporozoite antigen. Since CS specific T and B cells are both elicited by the vaccine, natural exposure to the antigen could help maintain the level of immunity during the entire period of exposure.

Another strategy to improve the immunogenicity of peptide vaccines is to stimulate the T and B cell responses artificially by adding interleukin 2 to the vaccine. Results with myoglobin indicate that genetic nonresponsiveness can be overcome by appropriate doses of interleukin 2 (109). The same effect was found for peptides derived from malaria proteins (110; K. Akaji, D. T. Liu, and I. J. Berkower, *unpublished results*). It is not yet clear whether this effect is based on immunizing T cells or whether low T cell responsiveness is overcome by a direct effect on B cells. In the former case, active immunity could result once specific T and B

cell clones are expanded, even in individuals who would otherwise be nonresponders.

One of the most important possible uses of peptide antigens is as synthetic vaccines. However, even though it is possible to elicit with synthetic peptides anti-influenza antibodies to nearly every part of the influenza hemagglutinin (73), antibodies that neutralize viral infectivity have not been elicited by immunization with synthetic peptides. This may reflect the fact that antibody binding by itself often does not result in virus inactivation. Viral inactivation occurs only when antibody interferes with one of the steps in the life cycle of the virus, including binding to its cell surface receptor, internalization, and virus uncoating within the cell. Apparently, antibodies can bind to most of the exposed surface of the virus without affecting these functions. Only those antibodies that bind to certain "neutralizing" sites can inactivate the virus. In addition, as in the case of the VP1 coat protein of poliovirus, certain neutralizing sites are found only on the native protein and not on the heat-denatured protein (111). Thus not only the site but also the conformation that is bound by the antibodies may be important for the antibody to inactivate the virus. These sites may often be assembled topographic sites not mimicked by peptide segments of the sequence. Perhaps binding of an antibody to such an assembled site can alter the relative positions of the component subsites so as to induce an allosteric neutralizing effect. Alternatively, antibodies to such an assembled site may prevent a conformational change necessary for activity of the viral protein.

One method of mapping neutralizing sites is based on the use of neutralizing monoclonal antibodies. The virus is grown in the presence of neutralizing concentrations of the monoclonal antibody, and virus mutants are selected for the ability to overcome antibody inhibition. These are sequenced, revealing the mutation that permits "escape" by altering the antigenic site for that antibody. This method has been used to map the neutralizing sites of influenza hemagglutinin (112) as well as poliovirus capsid protein VP1 (113). The influenza escaping mutations are clustered to form an assembled topographic site, with mutations distant from each other in the primary sequence of hemagglutinin but brought together by the three-dimensional folding of the native protein. At first, it was thought that neutralization was the result of steric hindrance of the hemagglutinin binding site for the cell surface receptor of the virus (114). However, similar work with poliovirus reveals that neutralizing antibodies that bind to assembled topographic sites may inactivate the virus at less than stoichiometric amounts, when at least half of the sites are unbound by antibody (115). The neutralizing antibodies all cause a conformational change in the virus, which is reflected in a change in the isoelectric point of the particles from pH 7 to pH 4 (113,116). Antibodies that bind without neutralizing do not cause this shift. Thus an alternative explanation for the mechanism of antibody-mediated neutralization is the triggering of the virus to self destruct. Perhaps the reason that neutralizing sites are clustered near receptor-binding sites is that occupation of such sites by antibody mimics events normally caused by binding to the cellular receptor, causing the virus to prematurely trigger its cell entry mechanisms. However, in order to transmit a physiologic signal, the antibody may need to bind viral capsid proteins in the native conformation (especially assembled topographic sites), which antipeptide antibodies may fail to do. Antibodies of this specificity are similar to the viral receptors on the cell surface, some of which have been cloned and expressed without their transmembrane sequences as soluble proteins. The soluble recombinant receptors for poliovirus (117) and HIV-1 (118–120) exhibit high-affinity binding to the virus and potent neutralizing activity *in vitro*. Th HIV-1 receptor, CD4, has been combined with the human immunoglobulin heavy chain in a hybrid protein CD4–Ig (121), which spontaneously assembles into dimers and resembles a monoclonal antibody, in which the binding site is the same as the receptor-binding site for HIV-1. In these recombinant constructs, high-affinity binding depends on the native conformation of the viral envelope glycoprotein gp120.

For HIV-1, two types of neutralizing antibodies have been identified. The first type binds a continuous or segmental determinant, the "V3 loop" sequence between amino acids 296 and 331 of gp120 (122). Antipeptide antibodies against this site can neutralize the virus (123). However, because this site is located in a highly variable region of the envelope, these antibodies tend to neutralize a limited range of viral variants. A second type of neutralizing antibody binds at or near the CD4 receptor-binding site of gp120 (124–126). These neutralizing antibodies, which are commonly found in the sera of infected patients, are specific for a broad range of HIV-1 variants, possibly due to conserved sequences around the CD4 binding site (127). Since the shared neutralizing determinant is an assembled topographic site, dependent on the native conformation of the protein (128), a prospective gp120 vaccine would need to be in the native conformation to be able to elicit these antibodies.

ANTIGENIC DETERMINANTS RECOGNIZED BY T CELLS

Mapping Antigenic Structures

Studies of T cell specificity for antigen were motivated by the fact that the immune response to protein antigens is regulated at the T cell level. A hapten, not immunogenic by itself, will elicit antibodies only when coupled to a protein that elicits a T cell response in that animal. This ability of the protein component of the conjugate to